Human Single-Chain Antibodies Reactive with Native Chondroitin Sulfate Detect Chondroitin Sulfate Alterations in Melanoma and Psoriasis

Toon F.C.M. Smetsers,* Els M.A. van de Westerlo,* Gerdy B. ten Dam,* Ingrid M. Overes,* Joost Schalkwijk,† Goos N.P. van Muijen,‡ and Toin H. van Kuppevelt*

*Department of Biochemistry, †Department of Dermatology and ‡Department of Pathology, University Medical Centre Nijmegen, NCMLS, HB Nijmegen, The Netherlands

Chondroitin sulfate (CS) belongs to the group of glycosaminoglycans (GAGs), which are linear polysaccharides, located in the extracellular matrix and on the cell surface. To study the structure and distribution of CS in human skin and skin disorders, we have selected antibodies using phage display technique against CS. Four unique human anti-CS single-chain antibodies were selected: IO3D9, IO3H10, IO3H12, and IO4C2. We determined their amino acid sequence and evaluated their CS reactivity using ELISA and immunohistochemistry. Antibodies were reactive with CS, but not with other GAGs except for IO4C2, which was also reactive with heparin. Antibody IO3D9 showed a strong reactivity with highly sulfated CS (CSE). All antibodies displayed a different staining pattern in rat kidney, indicating the recognition of unique CS epitopes. In normal skin, the papillary dermis but not the reticular dermis was strongly stained. Antibody IO3H12 also stained basal keratinocytes. We applied these antibodies to study CS expression and localization in melanoma and psoriasis. A strong immunoreactivity with the extracellular matrix of melanoma metastases could be observed for all four antibodies, while in atypical nevi a less extensive reactivity with only the papillary dermis was observed. In psoriatic lesions, CS could be observed in the papillary dermis and in the reticular dermis, whereas the specific location in the papillary dermis found in normal skin was completely lost. In conclusion, human phage-display-derived anti-CS antibodies have been selected, characterized, and applied to detect CS alterations in skin conditions. Altered CS composition was detected in melanoma and psoriasis.

Key words: glycosaminoglycan/monoclonal antibodies/phage display/skin disease
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Glycosaminoglycans (GAGs) are complex negatively charged polysaccharides, which together with core proteins form proteoglycans. GAGs can be grouped into five classes by their basic disaccharide constitution. The different classes are hyaluronate (HA), chondroitin sulfate (CS), keratan sulfate (KS), heparan sulfate (HS)/heparin, and dermatan sulfate (DS). GAGs are involved in processes such as cell signaling, cell adhesion, and cell migration (Tumova *et al*, 2000). This is mediated by the ability of GAGs to specifically bind and modulate a large number of proteins (Conrad, 1998). Proteins are bound as a result of chemical modifications superimposed on the saccharide backbone. GAGs regulate four aspects with respect to the proteins they bind: localization, modification, stabilization, and activation (Tumova *et al*, 2000). For instance, growth factors can be stored and activated by GAGs in the extracellular matrix and at the cell surface, thus controlling cell proliferation, differentiation, and regulation of synthesis and remodeling of the extracellular matrix (Taipale and Keski-Oja, 1997).

The structure of GAGs has been subject to detailed investigations. Biochemical techniques including sequencing have been used to study GAG structure (Lindahl *et al*, 1998; Turnbull *et al*, 1999; Venkataraman *et al*, 1999). Oligosaccharide sequencing techniques, however, are complicated and only small oligosaccharides can be characterized. Therefore, other approaches have been probed, including immunological methods. Antibodies were selected that react with HS (David *et al*, 1992; van den Born *et al*, 1995), CS (Couchman *et al*, 1984; Caterson *et al*, 1990a; Sorrell *et al*, 1990), and KS (Caterson *et al*, 1983). Using antibodies and biochemical techniques, the presence of GAGs in human skin was determined (Sorrell *et al*, 1990). HS is mainly located in basement membranes (Andriessen *et al*, 1997), while CS is abundantly present in the papillary dermis, and less in the reticular dermis. The major chondroitin sulfate proteoglycan in skin is versican (Bernstein *et al*, 1995; Sorrell *et al*, 1999), which is associated with elastic fibers (Bernstein *et al*, 1995), and plays a role in

Abbreviations: CS, chondroitin sulfate; C-4S, chondroitin-4 sulfate; C-6S, chondroitin-6 sulfate; CSA, chondroitin sulfate A; CSC, chondroitin sulfate C; CSE, chondroitin sulfate E; DE, dermis; DS, dermatan sulfate; EP, epidermis; GAG, glycosaminoglycan; HS, heparan sulfate; scFv, single-chain variable fragment

cellular proliferation and tissue remodeling (Zimmermann *et al*, 1994).

The anti-GAG antibodies currently used are of IgM nature, which makes them difficult to handle. The phage display technique forms an alternative to the traditional way of antibody generation. Selection of antibodies by this *in vitro* method is fast. Furthermore, antibodies against self-antigens and poorly immunogenic targets, like CS, can be more easily selected. Because the sequences of the antibody genes are known, identification, optimization, and labeling of antibodies is easy. The cloned antibodies can be expressed as intrabodies to study GAG functions (Jenniskens *et al*, 2003). Antibody genes of a large number of species can be used including human, the latter being attractive for future therapeutic use. Using the phage display technique, a number of anti-HS/heparin antibodies have been selected (van Kuppevelt *et al*, 1998; Jenniskens *et al*, 2000; Dennissen *et al*, 2002; van de Westerlo *et al*, 2002). These antibodies react with different epitopes on the HS chains. In this study, we have used the phage display technique to specifically select human antibodies against CS. We have used these antibodies to study the *in situ* location of CS epitopes in normal skin and two skin disorders, melanoma and psoriasis.

Results

Selection and characterization of anti-CS antibodies Four rounds of panning using the phage display library "scFv library no. 1" (Nissim *et al*, 1994) were performed using immobilized CSC (containing mainly C-6S) to select antibodies reactive with CS. The phage titer increased during selection from 10^5 individual clones in the first round to 10^8 clones in the fourth round. Individual clones from the third and fourth selection rounds were induced to produce soluble antibodies, which were tested in ELISA for their binding towards immobilized CS. Of the five CS-positive clones from the third selection, three unique clones could be identified. All 11 CS-positive clones from the fourth selection were identical, but different compared to the clones from the third selection. Clone data are displayed in Table I. Antibodies IO3D9 and IO3H10 both belong to the V_H-1 family and have the same V_H germline segment (DP-7). Antibodies IO3H12 and IO4C2 both belong to the V_H-3 family, but have a different V_H germline segment, DP-46 and DP-54, respectively. These clones are unique and were not found in previous antibody selections to heparin and HS (Jenniskens *et al*, 2000; Dennissen *et al*, 2002; van de Westerlo *et al*, 2002). The antibody genes were subcloned into vector pUC 119 HIS VSV containing the VSV-tag. The use of the VSV-tag for immunofluorescence assays resulted in reduced background compared to the original c-myc-tag.

The antibodies were tested for their reactivity with different GAGs and polyanions immobilized onto an ELISA plate (Fig 1). All antibodies reacted with CSC and also with CSA. This is not surprising since both preparations contain C-6S and C-4S, although CSC is enriched for C-6S and CSA for C-4S. Since three of four antibodies showed a higher reactivity with CSC, it is likely that they have a preference for C-6S. The antibodies also reacted with highly sulfated chondroitin sulfate E (CSE). Antibody IO3D9 showed a high reactivity with CSE compared to CSA or CSC. Antibodies IO3H10, IO3H12, and IO4C2 bound to immobilized CSE to about the same extent as to CSC. This indicates that the IO3D9 epitope is different from the other CS epitopes. All antibodies reacted exclusively with CS except IO4C2, which also reacted with heparin in an ELISA assay (Fig 1). No reactivity was observed with any of the other investigated GAGs or polyanions. To further evaluate if the antibodies recognized different CS epitopes, immunohistochemistry was performed using rat kidney sections, which are a rich source of various GAG epitopes, with a specific location (Dennissen *et al*, 2002). Marked differences in staining pattern for all four antibodies were observed (Fig 2). All anti-CS antibodies reacted with the interstitial space and peritubular capillaries. No staining of the glomerulus was observed with antibodies IO3H10 and IO4C2 (Fig 2*A, D*), whereas IO3H12 reacted weakly (Fig 2*C*) and IO3D9 strongly (Fig 2*B*). Only antibody IO3H12 stained the brush border area of the tubules (Fig 2*C*). These data show that the staining pattern in kidney is unique for all four anti-CS antibodies, indicating that different CS epitopes are recognized by these antibodies.

Sensitive detection of CS by anti-CS antibodies in solution We also tested the reactivity of the anti-CS antibodies with CSC and HS from bovine kidney in solution (Fig 3). As a control we used IgM antibody CS-56, reactive with CS (Avnur and Geiger, 1984). Binding of all antibodies to immobilized CSC could be inhibited by CSC, but not by HS, indicating that, also in solution, the antibodies

Table I. Antibody selection and sequence information of scFv antibodies

Clone name	CDR3-region amino acid sequence	V_H family	V_H segment	Number of clones from third selection	Number of clones from fourth selection
IO3D9	GIKHRHTQ	1	DP-7	3	
IO3H10	AKRLDW	1	DP-7	1	
IO3H12	MKTRLDV	3	DP-46	1	
IO4C2	GKQRYS	3	DP-54		11

For each unique clone the CDR3-region amino acid sequence, the V_H family, and the V_H segment (DP nomenclature) (Tomlinson *et al*, 1992) are indicated. The CDR3 sequences are shown in single-letter amino acid abbreviations. The Q in italic represents the translation of an amber codon to glutamine in *E. coli* TG-1 *sup*E strain.

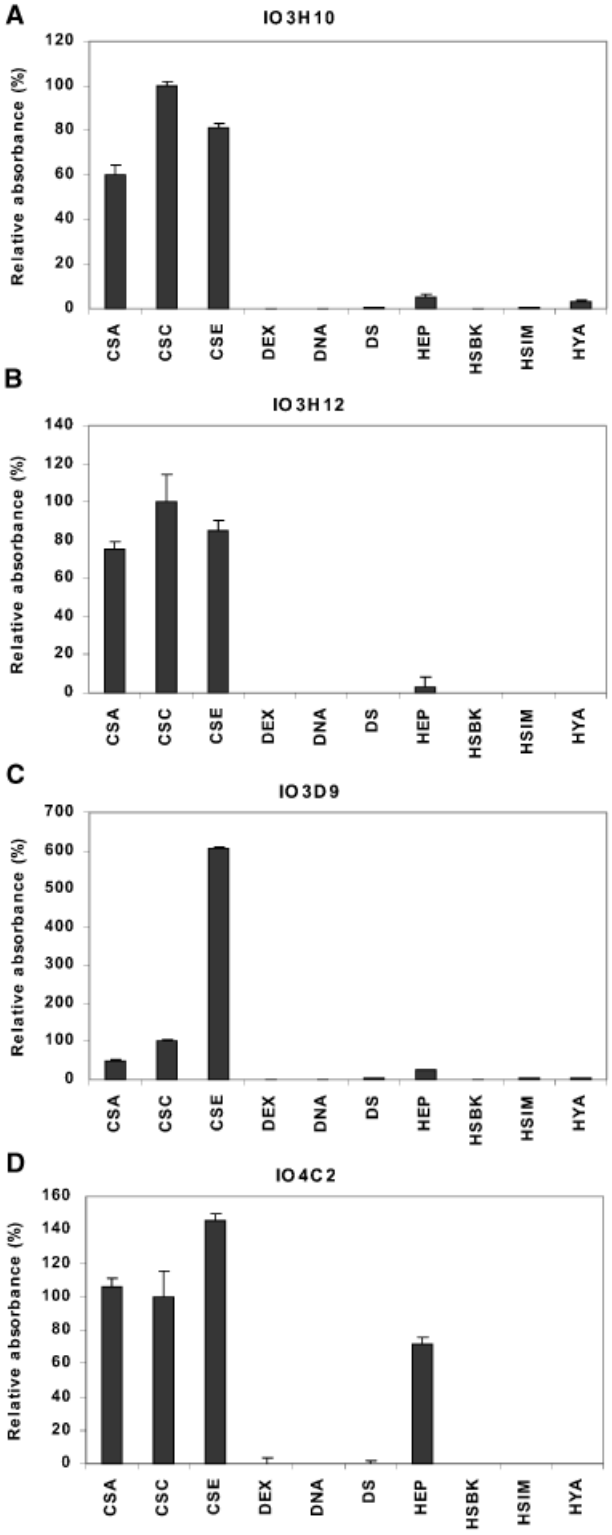

Figure 1
Reactivity of anti-CS antibodies with immobilized glycosaminoglycans. ELISA assay to determine the reactivity of the antibodies IO3H10 (*A*), IO3H12 (*B*), IO3D9 (*C*), and IO4C2 (*D*) towards immobilized glycosaminoglycans and other polyanions. The results are presented relative to the reactivity to CSC reactivity. CSA, chondroitin sulfate A (mainly C-4S); CSC, chondroitin sulfate C (mainly C-6S); CSE, chondroitin sulfate E, DEX, dextran sulfate; DNA, calf thymus DNA; DS, dermatan sulfate; HEP, heparin; HSBK, heparan sulfate from bovine kidney; HSIM, heparan sulfate from intestinal mucosa, HYA, hyaluronic acid. Note the high reactivity for CSE of antibody IO3D9. Values are given as mean ± SD (n = 3).

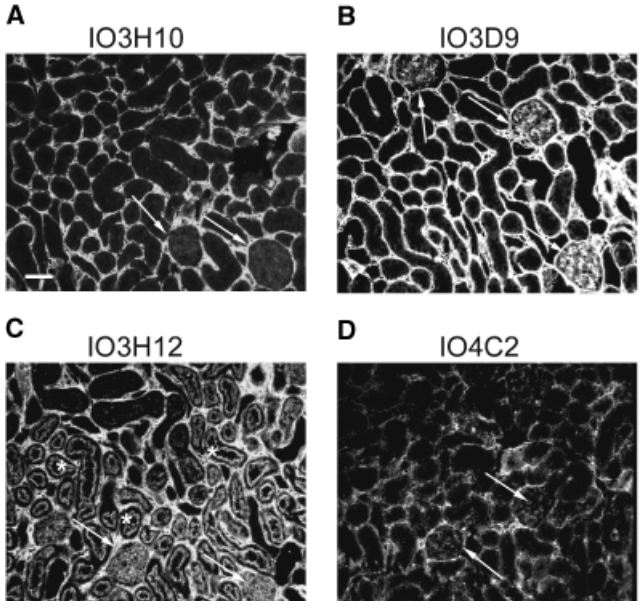

Figure 2
Evaluation of anti-CS antibodies using rat kidney immunoreactivity. Cryosections of rat kidney were incubated with antibodies and detected by a Cy3-labeled anti-VSV-tag antibody. Note the difference in staining pattern indicating the different CS epitopes detected by the different antibodies. Arrows mark the glomerulus, and asterisks mark the brush border. (*A*) IO3H10, (*B*) IO3D9, (*C*) IO3H12, (*D*) IO4C2. *Scale bar*: 100 μm.

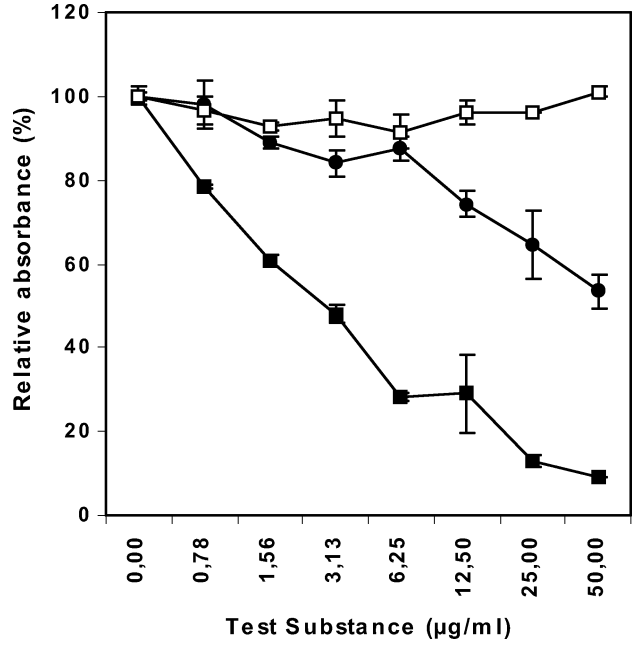

Figure 3
Reactivity of anti-CS scFv antibody IO3H10 and anti-CS IgM CS-56 with CSC and HS in solution. Anti-CS antibodies IO3H10 and CS-56 were added to CSC and HS preparations, and transferred to CSC-coated wells. Antibody binding of IO3H10 was detected using anti-VSV mouse monoclonal antibody P5D4, followed by alkaline phosphatase-conjugated rabbit anti-mouse IgG. Anti-CS-56 was detected using an alkaline phosphatase-conjugated rabbit anti-mouse IgM. Enzymatic activity was measured using *p*-nitrophenyl phosphate as a substrate. Values represent the reactivity, relative to antibody binding without the addition of a test substance, which was set to 100%. (■) CSC (IO3H10), (□) HS (IO3H10), (•) CSC (CS-56). Values are given as mean ± SD (n = 3).

specifically bind CS. The IC_{50} dose, the amount of CS resulting in 50% inhibition of binding, was about 1 μg per mL for all anti-CS antibodies and 50 μg per mL for CS-56, indicating that the phage display-derived anti-CS antibodies allow a sensitive and specific detection of CS in solution.

Next we determined the binding capacity of the antibodies with different CS preparations in solution in competition ELISA. The results for antibody IO3H10 are shown in Fig 4. For all anti-CS antibodies, similar results were obtained. Binding of immobilized CSC could be inhibited with low concentrations of CSE, moderate concentrations of CSA, and relatively high concentrations of CSC. No inhibition with HS was observed (Figs 3 and 4). The IC_{50} dose of CSE is smaller than 0.1 μg per mL, for CSC about 1 μg per mL, and for CSA about 10 μg per mL. This indicates that the preference for antibody binding to the different CS preparations in solution is in the following order: CSE > CSC > CSA.

CS epitopes in normal skin and psoriasis Using the CS-reactive antibodies, we investigated the expression and location of CS epitopes in normal skin. Immunoreactivity of the anti-CS antibodies with the papillary dermis, blood vessels, and fibers in the dermis was observed (Fig 5*A, B*). Anti-CS antibody staining in the papillary dermis overlaps with collagen type IV staining (Fig 5*C, D*), indicating that CS is located in the basement membrane. Anti-CS antibody staining also co-localizes with elastin fibers in the dermis, as indicated by autofluorescence of elastin (Fig 5*E, F*).

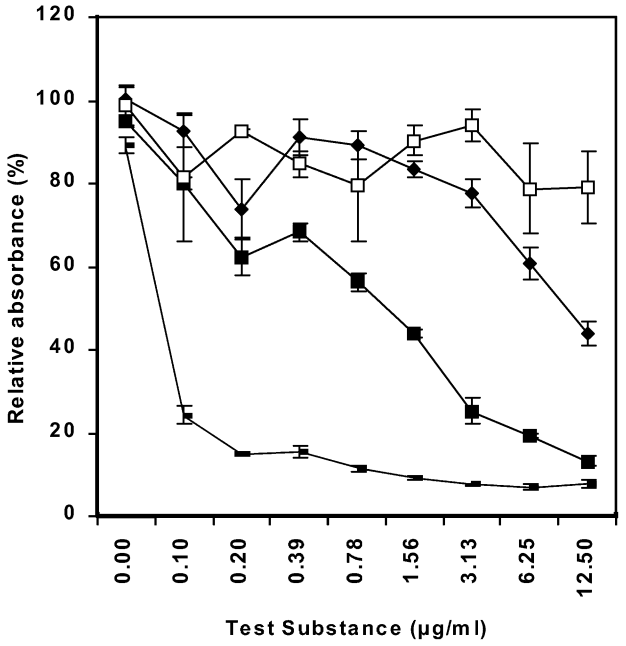

Figure 4
Reactivity of anti-CS antibodies with different CS preparations in solution. Anti-CS antibody IO3H10 was added to CSA, CSC, CSE, or HS preparations, and then transferred to CSC-coated wells. Antibody binding of IO3H10 was detected using anti-VSV mouse monoclonal antibody P5D4, followed by alkaline phosphatase-conjugated rabbit anti-mouse IgG. Enzymatic activity was measured using *p*-nitrophenyl phosphate as a substrate. Values represent the reactivity in percent relative to antibodies without the addition of a test substance. (◆) CSA, (■) CSC,: −CSE, (□) HS. Values are given as mean ± SD (n = 3).

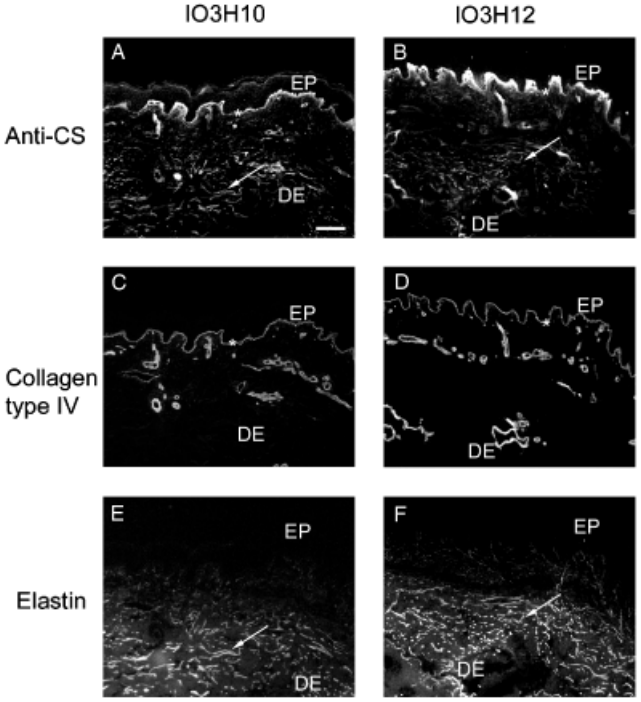

Figure 5
Location of CS epitopes in normal skin. Cryosections of normal skin were incubated with anti-CS antibodies IO3H10 (*A, C, E*) and IO3H12 (*B, D, F*) together with a collagen type IV antibody on the same section. Elastin was detected by its autofluorescence (*E, F*). Bound antibodies were visualized using fluorescently labeled secondary antibodies. CS epitopes are detected in the papillary dermis and co-localize with collagen type IV staining (see the asterisk) that is present in the basement membrane. Staining of antibodies IO3H10 and IO3H12 also correlates with the staining of elastic fibers (*E, F*) (marked by arrows). *Scale bar*: 100 μm.

All scFv anti-CS antibodies and antibody CS-56 showed similar results on different biopsies (n = 7) (Fig 6*A, C, E, G*). Antibody IO3H12, but not the other antibodies, also stained basal keratinocytes (Fig 6*C*, big arrow). In biopsies of psoriatic skin (Fig 6*B, D, F, H*), the prominent CS location in the papillary dermis was lost and a more diffuse staining extending into the reticular dermis was observed (Fig 6*B, D, F, H*). This was noticed in all cases investigated (n = 7). Also the keratinocyte staining by antibody IO3H12 was lost in psoriatic lesions (Fig 6*D*). The IgM antibody CS-56 showed identical CS location in normal and psoriatic skin as the phage-display-derived antibodies, and does not show keratinocyte staining (Fig 6*G*). An irrelevant control single-chain antibody TSCO1 (van de Westerlo *et al*, 2002) did not stain the dermis and papillary dermis in normal and psoriatic skin (Fig 6*I, J*), indicating that the reactivity of the antibodies is specific. These data indicate that in psoriatic skin, marked differences in CS epitope location occur compared to normal skin.

CS epitopes in melanoma We also tested the presence of CS epitopes in different stages of melanocytic tumor development using the antibodies. All single-chain antibodies showed a marked reactivity with melanoma metastases, indicating a high abundance of the CS epitopes (Fig 7*A, C*). Antibody CS-56 showed similar results (Fig 7*E*).

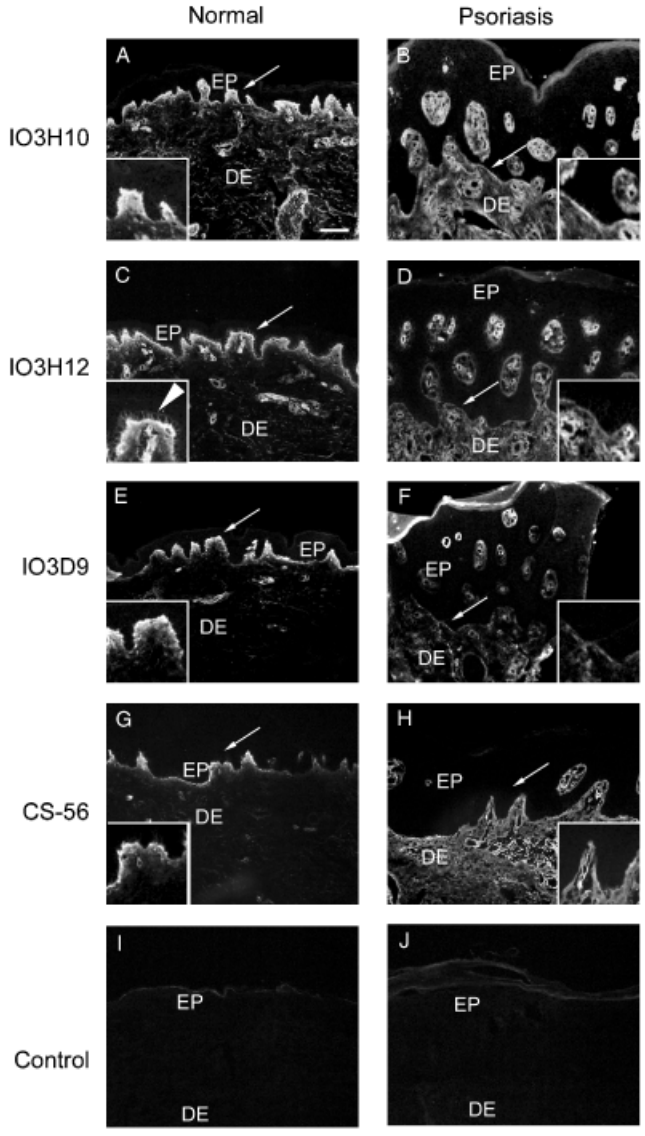

Figure 6
Detection of CS epitopes in normal and psoriatic skin. Cryosections of normal (*A, C, E, G, I*) and psoriatic skin (*B, D, F, H, J*) were incubated with anti-CS antibodies IO3H10 (*a, b*), IO3H12 (*c, d*), IO3D9 (*e, f*), CS-56 (*G, H*) and control single-chain antibody TSCO1 (*I, J*). Bound antibodies were visualized using fluorescently labeled secondary antibodies. Note that in the dermis (DE) of normal skin, CS epitopes are detected in the papillary dermis, while in psoriatic skin they are equally distributed in the reticular dermis, as well as the papillary dermis. Basal keratinocytes are stained by antibody IO3H12 (*C*, marked by a big arrow) in the epidermis (EP). The area of interest is marked by an arrow and enlarged in the lower corner. Basal keratinocyte staining is not detected in psoriatic skin (*D*). In contrast to IO3H12, antibodies IO3H10, IO3D9, and CS-56 do not stain basal keratinocytes in normal and psoriatic skin. Control single-chain antibody TSCO1 does not stain the papillary dermis. *Scale bar*: 100 μm.

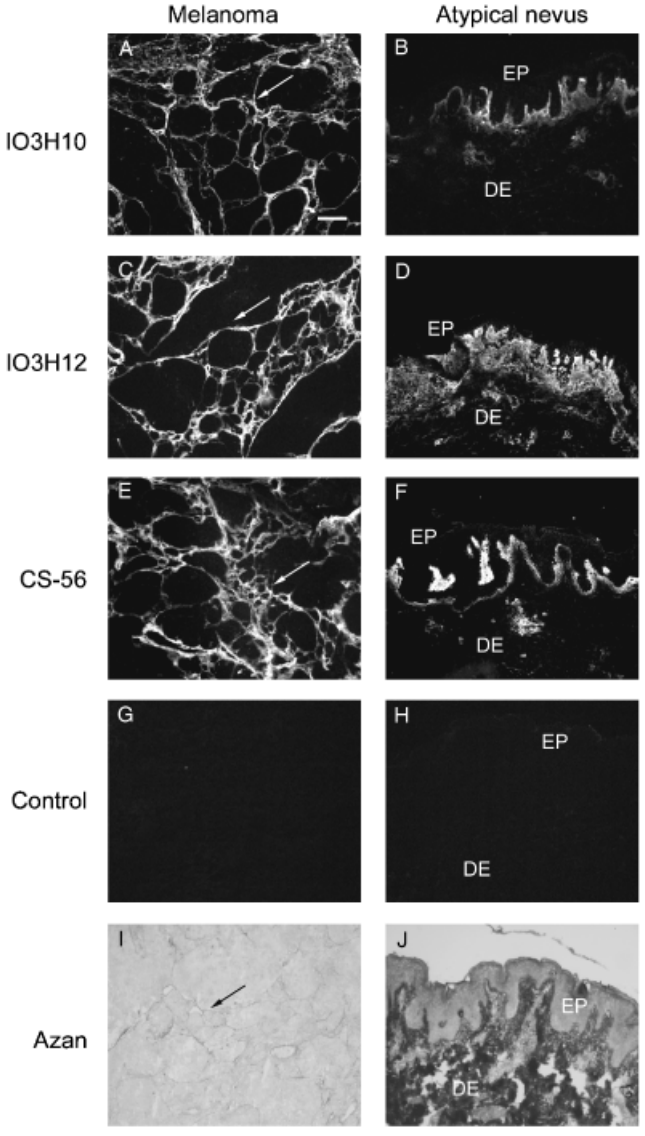

Figure 7
Detection of CS epitopes in different stages of melanocytic tumor development. Reactivity of anti-CS antibodies IO3H10 (*A, B*), IO3H12 (*C, D*) CS-56 (*E, F*), and control antibody TSC01 (*G, H*) with a human melanoma metastasis (*A, C, E, G*) and atypical nevus (*B, D, F, H*). Cryosections were incubated with anti-CS antibodies. Bound antibodies were visualized using fluorescently labeled secondary antibodies. An Azan staining marks the ECM in melanoma (*I*) and the location of the dermis in dysplastic nevus (*J*). Arrows mark the ECM in melanoma metastases. Intense staining of ECM in melanoma metastases was observed (*A, C, E*). In the dermis (DE) of atypical nevi, the staining of the papillary dermis was more intense than in the reticular dermis (*B, D, F*). The epidermis (EP) is not stained. *Scale bar*: 100 μm.

To show the location of the ECM and the papillary dermis, an Azan staining was performed (Fig 7*I, J*). The antibodies reacted predominantly with ECM surrounding nests of melanoma cells. In atypical nevi, the papillary dermis was stained (Fig 7*B, D, F*). The papillary dermis showed a much brighter staining than the reticular dermis, as was observed in normal skin biopsies. We used single-chain antibody TSCO1 as a specificity control. This antibody did not stain melanoma or atypical nevus sections (Fig 7*G, H*).

To confirm GAG specificity of the anti-CS antibodies, we performed digestions with chondroitinase ABC on tissue sections (Fig 8). The reactivity of antibodies IO3H10, IO3H12, and IO3D9 was abolished by digestion of kidney, melanoma, atypical nevus psoriatic skin, and normal skin with chondroitinase ABC, indicating that the antibodies specifically detect GAGs. Staining of antibody IO4C2 is reduced but not abolished by digestion with chondroitinase ABC.

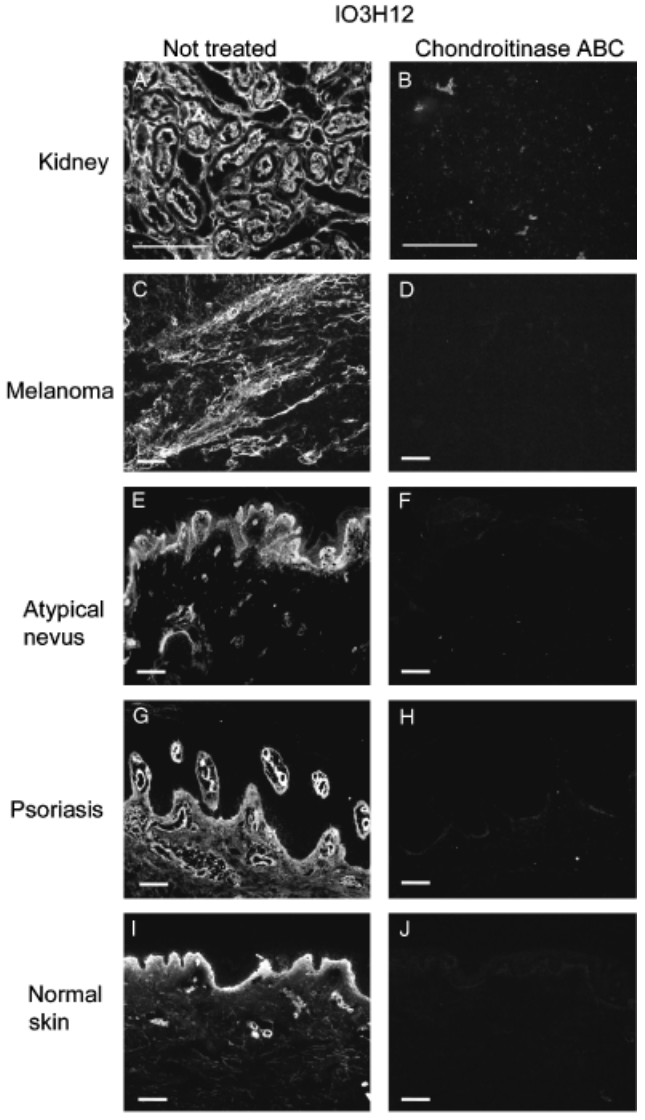

Figure 8
Determination of anti-CS antibody specificity by chondroitinase treatment. Reactivity of anti-CS antibody IO3H12 was determined after treatment with chondroitinase ABC (*B, D, F, H, J*) or with chondroitinase buffer alone (*A, C, E, G, I*) on rat kidney (*a, b*), human melanoma (*C, D*), human atypical nevus (*E, F*), human psoriatic skin (*G, H*), and human normal skin (*I, J*). Cryosections were incubated with or without chondroitinase ABC and subsequently with anti-CS antibody IO3H12. Bound antibodies were visualized using fluorescently labeled secondary antibodies. Note that the staining of antibody IO3H12 is completely lost in all tissue sections after treatment with chondroitinase ABC. *Scale bar*: 100 μm.

Discussion

CS is a class of GAGs, which are linear polysaccharides located in the extracellular matrix and on cells. The CS backbone is made up of alternating β1,3 and β1,4 glycosidic-linked glucuronic acid (GlcUA) and *N*-acetyl galactosamine (GalNAc) residues. CS can carry either a 4-sulfate and/or a 6-sulfate on the GalNAc moiety, and a 2-sulfate on the GlcUA moiety (Calabro *et al*, 2000). A 3-*O*-sulfate on GlcUA has also been demonstrated. Given this structural variability, there are theoretically over a million possibilities for a decasaccharide. This makes CS, with an average length of 50–100 disaccharides, a hypervariable molecule. The selective binding of proteins to CS is dictated by the sulfation pattern. Interferon gamma (Fernandez-Botran *et al*, 1999; Hurt-Camejo *et al*, 1999), low-density lipoprotein (Kaplan and Aviram, 2000), tumor necrosis factor-stimulated gene-6 (TSG-6) (Park *et al*, 2000), growth factors like midkine (Zou *et al*, 2000), and ECM molecules like tenascin X (Elefteriou *et al*, 2001) are some examples of proteins that bind to CS. Highly sulfated CS (CSE), which is 4- and 6-sulfated, has the capacity to bind additional heparin-binding proteins like FGFs (Deepa *et al*, 2002). In contrast to CS–protein interactions, the binding of heparin and HS with proteins has been subject to a large number of studies (Bernfield *et al*, 1999; Capila and Linhardt, 2002). As a result, much is known about the structural diversity in HS and heparin responsible for protein binding. To analyze structural diversity of CS in skin and of CS alterations in skin pathology, we have selected antibodies to CSC, which mainly contains C-6S. Four antibodies—IO3H10, IO3D9, IO3H12, and IO4C2—were selected. The reactivity and V_H sequence of these anti-CS antibodies are quite different from previously selected anti-HS antibodies.

In this study we have investigated the binding of the antibodies to CSA (mainly 4-sulfated), CSC (mainly 6-sulfated), and CSE (both 4- and 6-sulfated). All four antibodies show a specific reactivity with CSA, CSC, and CSE. Surprisingly, only antibody IO3D9 shows a much higher binding capacity to immobilized CSE compared to the other antibodies. This shows that the nature of the IO3D9 epitope is quite different from the epitopes recognized by the other three antibodies. In solution, all antibodies have a preference for CSE above CSC (Fig 4). CSA is bound more weakly than CSC. The difference of reactivity to CSE in solution compared to immobilized CSE is probably related to differences in immobilization of the GAG preparations. All anti-CS antibodies recognize unique epitopes in native CS chains. Different distribution patterns in rat kidney and human skin, and a distinct immunoreactivity towards CS preparations illustrate that the anti-CS antibodies recognize structurally different CS epitopes.

The first anti-CS antibodies that were described in the literature detected chondroitinase-treated CS and react with an unsaturated uronic acid residue, which was formed after digestion (Couchman *et al*, 1984; Caterson *et al*, 1985). Antibodies recognizing unmodified CS have also been identified (Yamagata *et al*, 1987; Sorrell *et al*, 1990; Hemming *et al*, 1994), including polyclonal antibodies (Karamanos *et al*, 1995). These antibodies however have either not been characterized completely (Hemming *et al*, 1994) or CS specificity is not always proven. The most widely used and best characterized antibody is CS-56, reactive with C-4S and C-6S (Avnur and Geiger, 1984). Most antibodies that detect unmodified CS chains are mouse antibodies of the IgM subclass. This limits their possibilities. Because of the few specific antibodies that have been described, alternative antibodies detecting unmodified CS chains are clearly of use to study the structure of CS. The human scFv anti-CS antibodies described here can be used as tools to visualize structural diversity in CS, as was previously illustrated with other antibodies in various tissues and model systems (Avnur and Geiger, 1984; Caterson *et al*,

1985; Caterson *et al*, 1990b; Sorrell *et al*, 1990, 1993, 1999). Since the cDNA of the single-chain antibodies is available, antibodies can be produced and purified in large quantities. In addition, cells can be transfected with antibody cDNA and induced to produce antibodies. This opens new avenues to study cell biology of CS epitopes *in vitro* and *in vivo*, as was recently demonstrated for anti-HS antibodies (Jenniskens *et al*, 2003). In this study we have investigated CS immunoreactivity in normal skin and two skin diseases, psoriasis and melanoma.

In normal skin the investigated CS epitopes are mainly expressed in the papillary dermis, although antibody IO3H12 also stains the basal keratinocytes. The CS epitopes are located in the basement membrane, blood vessels, and around elastin fibers (Fig 5). The dermal expression of these antibodies is comparable to the expression of the traditional antibody CS-56 (Fig 6) and also to antibody 6C3 (Sorrell *et al*, 1990). Chondroitinase treatment followed by antibody detection indicates that C-4S expression is located in the epidermis and the entire dermis, with higher expression in the papillary dermis, while C-6S expression is restricted to the papillary dermis (Sorrell *et al*, 1990). An antibody that detects CS in basal keratinocytes (PG-4) like IO3H12 has also been described (Sorrell *et al*, 1999). Other anti-CS antibodies detect CS in the epidermis or reticular dermis (Sorrell *et al*, 1990). The expression of the proteoglycan versican is detected in the subepidermal region and associated with elastic fibers (Zimmermann *et al*, 1994; Sorrell *et al*, 1999), and is age dependent (Willen *et al*, 1991). Like IO3H12 also the basal keratinocytes are stained. The single-chain anti-CS antibodies show an expression that is focussed in the papillary dermis. Decorin also shows a higher expression in the papillary dermis (Schonherr *et al*, 1993); this proteoglycan however is expressed throughout the dermis. Therefore, the expression of the CS epitopes partially correlates with the expression proteoglycan core proteins.

We have detected alterations in the distribution of CS in psoriasis. All CS antibodies showed a loss of the focussed expression in the papillary dermis observed in normal skin. Instead, the entire dermis shows a diffuse staining in all psoriatic lesions investigated. No alterations of CS or CS proteoglycans have been described previously, but alterations of hyaluronan (Wells *et al*, 1991) and HS (Seyger *et al*, 1997) have been reported. For hyaluronan, a diffuse meshwork, comparable to the immunoreactivity pattern observed for the anti-CS antibodies, was observed in six of 16 psoriatic patients (Wells *et al*, 1991). A loss of immunoreactivity of two anti-HS antibodies, JM-13 and JM-403, was observed in psoriatic skin (Seyger *et al*, 1997). This clearly indicates that in psoriatic lesions alterations in GAGs occur. It has been suggested that disturbed glycosaminoglycan location and expression in psoriatic lesions leads to malfunction of the basement membrane and protein leakage, thereby contributing to the disease process (Verschoore *et al*, 1990).

In melanoma metastasis the CS epitope expression detected by our anti-CS antibodies is high without exception (Fig 7), especially in ECM structures. In atypical nevi, the expression is restricted to the papillary dermis and blood vessels in the dermis and is not significantly different from that in normal skin. The presence of high amounts of CS in melanoma metastases was observed before in a previous study (Smetsers *et al*, 2003) and is in line with CS expression in other types of cancer (Yamori *et al*, 1988; Adany *et al*, 1990; Ricciardelli *et al*, 1997; Ricciardelli *et al*, 1999; Martins *et al*, 2000; Theocharis *et al*, 2000). The expression pattern does not resemble that of CD44 or HMW-MAA, which are expressed on the surface of nevus and melanoma cells (Manten-Horst *et al*, 1995; Smetsers *et al*, 2003). The expression of versican and melanoma-associated proteoglycan (mel-CSPG), however, is low in atypical nevi and high in melanoma, and also their expression is cell-associated (Touab *et al*, 2002, 2003).

A high amount of CS in tumors could allow tumors to store many specific CS-binding proteins, promoting tumor growth. Tumors treated with chondroitinase show reduced growth and metastasis formation, further indicating the involvement of CS (Denholm *et al*, 2001). The expression of melanoma chondroitin sulfate proteoglycan that is used as a marker in melanoma also indicates the role of CS in melanoma (Pluschke *et al*, 1996; Geiser *et al*, 1999). Finally, CS targeting liposomes can be used to therapeutically target drugs to metastases (Lee *et al*, 2002). The anti-CS antibodies selected, recognize CS that is located in ECM structures in between the tumor cells. These structures have a prognostic significance in uveal melanoma (Folberg *et al*, 1992) and may provide an additional way of fluid transport in cutaneous melanoma (Bittner *et al*, 2000). These data indicate the importance of CS in the extracellular matrix of melanoma and indicate that CS might be an important factor contributing to the malignant process.

Both melanoma and psoriasis show increased angiogenesis. For treatment, angiogenesis inhibitors are now tested in clinical trials (Kumar and Li, 2001). The angiogenesis inhibitor AE-941 shows promising effects for the treatment of psoriasis (Dupont *et al*, 1998; Sauder *et al*, 2002). This angiogenesis inhibitor contains shark cartilage extract, which contains large amounts of C-6S. In fact, the anti-CS antibodies in this study were selected against C-6S from shark cartilage. It is possible that treatment of psoriatic lesions with CS might complement for the local loss of certain CS epitopes or immobilize proteins, normally bound to CS in normal skin. The human scFv anti-CS antibodies described in this study may be useful in the purification and analysis of those CS saccharide epitopes that are involved in the pathogenesis of melanoma and psoriasis. This may lead to the synthesis of glycomimetics to be used for treatment. Also, since the antibodies are of human nature, immunotherapy may be an option.

We conclude that the four novel anti-CS antibodies described here are useful tools to study skin biology because they detect different CS epitopes and identify alterations of CS in melanoma and psoriasis.

Materials and Methods

The study was performed according to the ethical guidelines following the Declaration of Helsinki. It was approved by the University Medical Center Nijmegen and performed under Ministry of Health license GGO 01-075.

Antibody selection by phage display In this study the semi-synthetic single-chain variable fragment (scFv) library no. 1 (Nissim library) was used (Nissim *et al*, 1994). The library was stored as a glycerol stock in *Escherichia coli* (*E. coli*) TG1 bacteria (K12, Δ(*lac-pro*), *sup*E, *thi*, *hsd*Δ5/F′*tra*Δ36, *pro*A$^+$B$^+$, *lac*I^q, *lac*ZΔM15). This glycerol stock (50 μL) was inoculated in 50 mL of 2xTY medium containing 1% wt/vol casein, 1.6% wt/vol bacto tryptone (Gibco BRL, Life Technologies, Paisley, Scotland), 0.5% wt/vol NaCl. Bacteria were grown under constant rotation at 37°C until an A_{600} of 0.5 was reached. Then 50 μL 10^{12} plaque-forming units per mL helper phage VCS-M13 (Stratagene, La Jolla, California) was added to 10 mL of this culture and the phagemid-containing bacteria were infected with the helper phage by incubating for 30 min at 37°C without shaking. Cells were collected by centrifugation (10 min, 2500 × *g*, 22°C) and resuspended in 300 mL preheated (30°C) 2xTY medium with 100 μg per ml ampicillin (Sigma, St Louis, Missouri) and 25 μg per ml kanamycin (Life Technologies, Paisley, Scotland). Bacteria were cultured for 16 h at 30°C with shaking. Polystyrene 3 mL tubes (Greiner GmbH, Frickenhausen, Germany) were coated for 16 h with chondroitin sulfate C (CSC, 10 μg per mL) (Sigma). CSC mainly contains chondroitin 6-sulfate (C-6S), and also chondroitin 4-sulfate (C-4S). The tubes were washed with phosphate-buffered saline containing 0.1% Tween-20 (PBST). Non-specific binding sites were blocked by the addition of 2% non-fat dried milk (Marvel, Chivers Ireland, Dublin, Ireland) in PBST for 60 min under rotation, followed by washing five times with PBST. Bacteria were collected by centrifugation (10 min, 2500 × *g*, 4°C) and phages were precipitated by the addition of 10 mL PEG/NaCl to 40 mL of the supernatant for 30 min on ice. The phages were collected by centrifugation (30 min, 5000 × *g*, 4°C), supernatant was removed, and the pellet resuspended in 1 mL milliQ water. One microliter of phage suspension was kept for phage titration. The rest of the precipitated phages were transferred into the CS-coated tube. Phages were allowed to bind to the tube for 30 min under rotation, and then 90 min without rotation. The tube was washed 20 times with PBST, and subsequently 20 times with PBS. Bound phages were eluted by incubation in 1 mL 100 mM triethylamine for 10 min under rotation. The eluate was neutralized by the addition of 0.5 mL 1 M Tris/HCl (pH 7.3). This suspension, containing the selected phages, was used to infect 9 mL of TG1 bacteria ($A_{600} = 0.5$). Phage-containing samples before and after selection were also used to infect TG1 to establish the phage titer. Infection was performed 30 min at 37°C without rotation and 90 min with rotation. A sample of 100 μL of the infected bacteria was plated onto TYE-agar plates (TY with bacto agar containing 1% (wt/vol) glucose and 100 μg per ml ampicillin). To the culture infected with phages from CS-binding selection, ampicillin (100 μg per mL) was added and this culture was incubated with shaking for 60 min at 37°C. The culture was superinfected with 50 μL VSC-M13 helper phages for 30 min at 37°C without rotation. After superinfection and centrifugation (10 min, 2500 × *g*, 22°C), the bacterial pellet was resuspended in 50 ml 2 × TY medium with 100 μg per ml ampicillin, which was preheated at 30°C. The culture was incubated with shaking for 60 min at 37°C, and then 25 μg per mL kanamycin was added to select for bacteria containing the helper phage. This culture was incubated shaking for 16 h at 30°C to produce phages. Four consecutive rounds of selection were performed. After each selection round, the phage titer was determined. Phage suspensions taken before and after the selection were tested.

Induction of antibody production by individual clones Bacterial colonies were transferred to a flat-bottom 96-well plate (Greiner, Frickenhausen, Germany) containing 100 μL per well 2 × TY medium containing 100 μg per mL ampicillin and 1% (wt/vol) glucose. These plates were incubated for 16 h with shaking at 37°C. Two microliters of the bacterial suspensions were transferred to corresponding wells in a round-bottom 96-well plate (Greiner) containing 2 × TY (150 μL per well) with ampicillin (100 μg per mL) and 0.1% (wt/vol) glucose. These plates were incubated at 37°C with shaking until A_{600} reached about 0.5. The original plates were stored at −80°C containing 20% glycerol. To the round-bottom plates, IPTG was added to a final concentration of 1 mM and these plates were incubated for 16 h at 30°C under constant rotation.

Determination of antibody reactivity by ELISA A 96-well flat-bottom plate was incubated for 16 h with 100 μL of a 10 μg per mL test substance solution in coating buffer. The test substances were HS from bovine kidney (Seikagaku Kogyo, Tokyo, Japan) and HS from intestinal mucosa, heparin from porcine intestinal mucosa, DS from pig skin, chondroitin sulfate A (CSA) (mainly C-4S) from bovine trachea and chondroitin sulfate C (CSC) (mainly C-6S) from shark cartilage, dextran sulfate, calf's thymus DNA and hyaluronic acid from rooster comb (all from Sigma), and chondroitin sulfate E (CSE) (Seikagaku Kogyo). After washing, 50 μL of the supernatant of the IPTG-induced bacteria was mixed with 50 μL 2% (wt/vol) BSA in PBST in each well. Alternatively, a 10-fold diluted antibody preparation from the periplasm (prepared as described (van de Westerlo *et al*, 2002)) in 1% (wt/vol) BSA in PBS was used. Antibody TSCO1 (van de Westerlo *et al*, 2002) served as a negative control while antibody EW4G1 (van de Westerlo *et al*, 2002) served as a positive control in these experiments. To test antibody-binding properties in solution, which are independent of coating properties, we performed competition ELISA. For competition ELISA, antibody dilutions were incubated in C-6S-coated wells together with different concentrations of test substance as a competitor for 90 min at 22°C. The plates were washed three times with PBST. Since these antibodies contain a c-myc tag, we used this tag for the detection of antibody binding. A hybridoma supernatant of the anti-c-myc tag mouse monoclonal antibody 9E10 (Boehringer Mannheim, Mannheim, Germany), diluted 2-fold in 2% (wt/vol) BSA in PBST, was added to the wells (100 μL per well). Also antibodies with a VSV-tag were used (see the section on subcloning). For the detection of bound VSV-tagged antibody, a hybridoma supernatant of anti-VSV-tag mouse monoclonal antibody P5D4, diluted 2-fold in 2% (wt/vol) BSA in PBST, was added (100 μL per well). The plates were incubated for 90 min at 22°C and washed three times with PBST. Then 1000-fold-diluted alkaline phosphatase-conjugated rabbit anti-mouse IgG (Dakopatts, Glostrup, Denmark) in 1% (wt/vol) BSA in PBST was added (100 μL per well) and the plates were incubated for 60 min at 22°C. The plates were washed three times with PBST and three times with PBS. Enzyme activity was detected the addition of 100 μL per well of 1 mg per mL 4-nitrophenylphosphate disodium salt in a solution of 1 mM diethanolamine, 0.5 mM $MgCl_2$, pH 9.8. Absorbance was measured using an ELISA reader at a wavelength of 405 nm.

Sequence analysis of the antibody region of the selected clones Phagemid DNA was purified from the bacterial pellet from an overnight culture of the selected clones by using the QIA prep spin mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The DNA was stored at −20°C. Sequencing of the antibody genes was performed on an ABI Prism 3700 DNA analyser (Applied Biosystems, Foster City, California) using primers PelBseq (5′-CCGCTGGATTGTTATTACTC-3′) or For Link Seq RIC (5′-GCCACCTCCGCCTGAACC-3′) and the BigDye terminator kit (Applied Biosystems) according to the manufacturer's instructions. V_H families and V_H segment (DP nomenclature) (Tomlinson *et al*, 1992) were determined by comparison with known V_H sequences using DNAPLOT software on http://www.mrc-cpe.cam.ac.uk/.

Subcloning of the antibody sequence into pUC119 HIS VSV vector The antibody coding sequence was digested from the vector using restriction enzymes *Not*I and *Nco*I (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and ligated into a similarly digested vector pUC119 (His)8 VSV containing a His(8)-tag and a VSV-tag. The sequence was verified as described above.

Preparation of antibody-containing periplasmic fractions An overnight culture of the different bacterial clones was diluted 100-fold in 500 mL sterile 2 × TY medium containing ampicillin (100 μg per mL) and 0.1% (wt/vol) glucose and incubated shaking at 37°C until the A_{600} reached 0.6. Then IPTG was added to a final concentration of 1 mM to induce antibody production. The cultures were incubated for 3 h at 30°C, cooled on ice for 15 min, and then centrifuged for 10 min at 2500 × *g* at 4°C. Cells were resuspended in 5 mL of a solution containing 200 mM sodium borate, 160 mM NaCl and EDTA-free protease inhibitors (Boehringer Mannheim, Mannheim, Germany), and centrifuged for 20 min at 5000 × *g* at 4°C. After filtration through a 0.45 μm filter, the fractions were dialyzed for 16 h against PBS at 4°C and stored in small aliquots at −20°C.

Immunohistochemistry Seven normal skin biopsies, seven psoriatic skin biopsies, five human melanoma metastases, and three atypical nevi were frozen in liquid nitrogen and stored at −80°C. Organs of adult Wistar rats were frozen. Cryosections of 5 μm were made, dried, and stored at −80°C until use. Before use, the cryosections were thawed, air dried for 1 h, and rehydrated with PBS. Occasionally, cryosections were subjected to enzyme digestion with chondroitinase ABC (Sigma), 1 IU per mL in 25 mM Tris/HCl, pH 8.0, to digest dermatan sulfate and C-6S and C-4S. Alternatively chondroitinase C (Sigma) was used to digest C-6S only.

Sections were incubated with the antibodies in PBS containing 1% BSA. Periplasmic fractions were diluted 10-fold, anti-CS-antibody CS-56 (Sigma) was used at a 50-fold dilution, and goat anti-collagen type IV (Southern Biotechnology Associates, Birmingham, Alabama) was used at a 20-fold dilution. Sections were washed three times for 5 min with PBS and subsequently incubated with secondary antibodies diluted in PBS containing 1% BSA. A 500-fold-diluted Cy3-conjugated anti-VSV-tag antibody P5D4 (Sigma) was used for the detection of VSV-tagged single-chain antibodies, a 100-fold-diluted goat anti-mouse IgM FITC (Sigma) was used for the detection of bound CS-56, and a 100-fold donkey anti-goat Alexa 488 (Molecular Probes Inc., Eugene, OR) was used for the detection of collagen type IV. Sections were washed again three times and fixed with methanol. Next the sections were embedded in Mowiol (Calbiochem, La Jolla, California) and immunoreactivity was visualized using an Axioscope fluorescence microscope equipped with a Nikon DXM1200 digital camera (Nikon, Tokyo, Japan).

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Address correspondence to: Dr T.H. van Kuppevelt, University Medical Centre Nijmegen, NCMLS, Department of Biochemistry, PO Box 9101, 6500 HB Nijmegen, The Netherlands. Email: a.vankuppevelt@ncmls.kun.nl

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